A *Dictyostelium discoideum* Mutant That Missorts and Oversecretes Lysosomal Enzyme Precursors Is Defective in Endocytosis

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Abstract. A mutant strain of *Dictyostelium discoideum*, HMW570, oversecretes several lysosomal enzyme activities during growth. Using a radiolabel pulse-chase protocol, we followed the synthesis and secretion of two of these enzymes, α-mannosidase and β-glucosidase. A few hours into the chase period, HMW570 had secreted 95% of its radiolabeled α-mannosidase and 86% of its radiolabeled β-glucosidase as precursor polypeptides compared to the secretion of <10% of these forms from wild-type cells. Neither α-mannosidase nor β-glucosidase in HMW570 were ever found in the lysosomal fractions of sucrose gradients consistent with HMW570 being defective in lysosomal enzyme targeting. Also, both α-mannosidase and β-glucosidase precursors in the mutant strain were membrane associated as previously observed for wild-type precursors, indicating membrane association is not sufficient for lysosomal enzyme targeting. Hypersecretion of the α-mannosidase precursor by HMW570 was not accompanied by major alterations in N-linked oligosaccharides such as size, charge, and ratio of sulfate and phosphate esters. However, HMW570 was defective in endocytosis. A fluid phase marker, [^{3}H]dextran, accumulated in the mutant at one-half of the rate of wild-type cells and to only one-half the normal concentration. Fractionation of cellular organelles on self-forming Percoll gradients revealed that the majority of the fluid-phase marker resided in compartments in mutant cells with a density characteristic of endosomes. In contrast, in wild-type cells [^{3}H]dextran was predominately located in vesicles with a density identical to secondary lysosomes. Furthermore, the residual lysosomal enzyme activity in the mutant accumulated in endosomal-like vesicles. Thus, the mutation in HMW570 may be in a gene required for both the generation of dense secondary lysosomes and the sorting of lysosomal hydrolases.

LYSOSOMAL enzymes in most cell types are synthesized on membrane-bound polysomes as precursor polypeptides. These precursor peptides are targeted to lysosomes using phosphomannosyl recognition markers and proteolytically processed to mature forms (for review see von Figura and Hasilik, 1986). In many cases, a small percentage of the precursor escapes targeting and is instead secreted constitutively in the precursor form (Hasilik and Neufeld, 1980*a*). If the targeting mechanism is disrupted as is the case in the human genetic disorder, I-cell disease, a much larger percentage of the lysosomal enzyme precursors are secreted (Hasilik and Neufeld, 1980*a*; Kornfeld, 1986).

The synthesis, transport, and processing of three lysosomal enzymes from *Dictyostelium discoideum* have been studied extensively (for review see Cardelli and Dimond, 1988). α-Mannosidase is synthesized on membrane-bound polysomes as a 140-kD precursor (Cardelli et al., 1986*b*) that is processed, with a half-life of ~26 min, to mature lysosomally localized peptides of 60 and 58 kD (Mierendorf et al., 1985) through an 80-kD intermediate (Pannell et al., 1982). β-Glucosidase is also synthesized on membrane-bound polysomes as a high molecular mass precursor of 105 kD (Cardelli et al., 1987), however, it is very rapidly processed to a 103-kD intermediate ($t_{1/2}$ = 10 min) that is, in turn, processed to the 100-kD mature polypeptide (Cardelli et al., 1986*a*). Acid phosphatase is synthesized as a 56-kD precursor polypeptide which is also cleaved rapidly to a 55-kD mature form (Bush and Cardelli, 1989). α-Mannosidase, β-glucosidase, and acid phosphatase are cotranslationally glycosylated and contain 20, 11, and 12 kD of N-linked oligosaccharides, respectively. In addition, the glycosylated precursor polypeptides are membrane associated while the mature forms are soluble (Mierendorf et al., 1985; Cardelli

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et al., 1986*a*, 1987). The enzymes are transported at nonuniform rates to the Golgi complex (Cardelli et al., 1986*a*) where additional modifications occur to the N-linked carbohydrate side chains (see below). 1–10% of the precursors are secreted constitutively (Mierendorf et al., 1985; Cardelli et al., 1986*a*; Bush and Cardelli, 1989). Finally, the mature lysosomal enzymes are secreted with different kinetics from lysosomal compartments when cells are starved for nutrients (Dimond et al., 1981), although all three lysosomal enzymes are colocalized to the same population of lysosomal vesicles (Bush and Cardelli, 1989).

We and others have shown that the initial proteolytic processing events occur in a prelysosomal compartment (Wood and Kaplan, 1985; Richardson et al., 1988). Furthermore, a small number of serine/cysteine proteinase inhibitors disrupt both proteolytic processing and lysosomal enzyme targeting, but have no effect on intravesicular pH (Richardson et al., 1988; Cardelli et al., 1989). Intracellular sorting, however, is not disrupted in cells treated with high concentrations of the weak base, ammonium chloride, suggesting that transport of enzymes to lysosomes is not dependent on acidic intracompartmental conditions (Cardelli et al., 1989).

Oligosaccharide modifications on *D. discoideum* lysosomal enzymes, like mammalian enzymes, are of the high mannose type and contain the lysosomal recognition marker, mannose-6-phosphate (Freeze, 1986; Freeze and Wolgast, 1986*a*,*b*). However, this modification is found as an unusual phosphomethyl diester (Freeze et al., 1983; Freeze and Wolgast, 1986*a*; Gabel et al., 1984). Interestingly, although the mannose-6-phosphate methyl ester (Man-P-OCH_3)[1] binds with high affinity to mammalian receptors (Freeze, 1985), no such receptors have been identified in *D. discoideum* (Cardelli et al., 1987). Furthermore, these oligosaccharides are characterized by the addition of mannose-6-sulfate (Freeze, 1985).

We are currently using biochemical and genetic approaches to determine the molecular mechanisms involved in the sorting of lysosomal enzymes. We have characterized the mutant, HMW570 (a member of a collection of over 70 secretion mutants), which secretes >90% of its α-mannosidase and β-glucosidase activities during axenic growth (Ebert, D. L., K. B. Jordan, and R. L. Dimond, manuscript submitted for publication). We demonstrate in this report that the oversecretion of enzyme activity is most likely due to inefficient targeting of newly synthesized enzyme precursors to lysosomes. N-linked oligosaccharides on HMW570 were of normal size, and were both phosphorylated and sulfated; however, this strain was defective in endocytosis and the generation of dense lysosomal vesicles. These results are consistent with a functional connection between the process of endocytosis, and the intracellular sorting of lysosomal enzymes in *D. discoideum*.

Materials and Methods

Organism

D. discoideum strains were grown axenically in TM medium (Free and Loomis, 1974) at 21°C in a rotary shaker water bath. In addition to the wild-type strains, Ax3 and Ax4, the α-mannosidase structural gene mutant M4 (Free and Loomis, 1974) and the secretory mutant HMW570 (derived from Ax3), were used. The wild-type strain Ax4 is a clonal derivative of Ax3 and is identical in all aspects of lysosomal enzyme biosynthesis and transport.

1. *Abbreviations used in this paper*: endo H, endoglycosidase H; Man-P-OCH_3, mannose-6-phosphate methyl ester; PNGase F, peptide:*N*-glycosidase F; QAE, quaternary ethyl amine.

Enzyme Assays

The enzymes α-mannosidase, β-glucosidase, *N*-acetylglucosaminidase, acid phosphatase, α-glucosidase I, and β-galactosidase 1 were assayed using *p*-nitrophenol substrates as described in Dimond et al. (1983).

Radioactive Labeling

To label proteins, exponentially growing cells were collected by centrifugation (1,000 *g* for 3 min) and resuspended to a titer of 10^7 cells/ml in fresh TM medium containing 500–800 μCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL; 1,200 μCi/mmol). After incubation at 21°C for the times indicated in the figure legends, the cells were quickly harvested by centrifugation and resuspended in 0.5% Triton X-100, or, if a chase period was required, resuspended to 10^7 cells/ml in fresh TM medium plus 10 mM methionine.

To label N-linked oligosaccharides, cells of each strain at 1×10^6 cells/ml were grown for 3 d in 5 ml of HL5 medium containing 600 μCi/ml of 2-[^{3}H]mannose (20 Ci/mmol). The cells were removed by centrifugation and the secreted α-mannosidase precursor and mature forms were isolated by immunoprecipitation as described below.

Immunoprecipitations

[^{35}S]Methionine-labeled α-mannosidase and β-glucosidase polypeptides were immunoprecipitated from crude cellular and secreted protein samples using the polypeptide-specific monoclonal antibodies: 2H9 for α-mannosidase (Mierendorf and Dimond, 1983) and mBG11 for β-glucosidase (Golumbeski and Dimond, 1986). Immunoprecipitations were carried out at 0°C similar to the method of Mierendorf et al. (1985). All samples were adjusted to a final concentration of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 2 mM methionine, 1 mM NaN_3, and 0.5% Nonidet P-40 (1×C) and incubated for 30 min with 0.1 vol of Pansorbin (Calbiochem-Behring Corp., San Diego, CA), followed by centrifugation at 10,000 *g* for 3 min in a microfuge. The supernatants were removed to fresh tubes, and excess monoclonal antibodies were added. After a 60–90-min incubation, excess Pansorbin was added, and incubation continued for another 60–90 min. The Pansorbin was collected by centrifugation and washed three times with 1×C. The final pellets were resuspended in 30 μl of gel sample buffer (150 mM Tris-HCl, pH 6.8, 2% SDS, 20% sucrose, 10% 2-mercaptoethanol, and 0.001% bromophenol blue).

The [^{3}H]mannose-labeled mature forms were precipitated by the addition of 3-μl aliquots of ascites preparations containing the monoclonal antibody 6H10, which recognizes mature forms only (Mierendorf and Dimond, 1983), followed by the addition of 50 μl of Pansorbin. The [^{3}H]mannose-labeled precursor form was similarly precipitated with the 2H9 antibody which recognizes both the precursor and the mature forms. After three sequential precipitations, the enzymes were eluted from the Pansorbin pellets in 100–200 μl of 1% SDS. An aliquot of each solubilized sample was analyzed by SDS-PAGE followed by fluorography.

Gel Electrophoresis

To prepare the immunoprecipitated samples for electrophoresis, the samples were heated to 95°C for 3 min and the Pansorbin removed by centrifugation. The eluted samples were subjected to SDS-PAGE (7.5% acrylamide) according to the procedure of Laemmli (1970). After electrophoresis, the gels were fixed in 10% trichloroacetic acid, treated with Enhance (New England Nuclear, Boston, MA), dried under vacuum, and exposed to Kodak XAR-5 x-ray film at −70°C.

Quantitative measurements of band intensities were determined by scanning fluorographs with an Ultrascan densitometer (LKB Instruments, Inc., Houston, TX). The recovery of radioactive precursor in the form of mature enzyme was near 100% at the end of all chases.

Subcellular Fractionation

Cellular organelles were fractionated on sucrose density-step gradients according to the procedure of Mierendorf et al. (1985). Pulse-labeled cells were mixed with M4 cells to a concentration of 3×10^8 cells/ml in 0.25 M sucrose-TKM (TKM is 50 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, and 5 mM DTT; the molarity refers to the molarity of the sucrose).

The M4 cells, which contain no immunoprecipitable forms of α-mannosidase, were added to facilitate breakage and handling. The cells were broken using 20 strokes of a Dounce homogenizer (Kontes Glass Co., Vineland, NJ), and nuclei and unbroken cells were removed by centrifugation at 1,000 *g* for 5 min. The pellet was resuspended and homogenized again. The Golgi apparatus (1.1–1.3 M sucrose), lysosomes (1.3–1.5 M sucrose), and the RER (1.5–1.8 M sucrose) were separated on a sucrose step-gradient as described (Mierendorf et al., 1985).

Endoglycosidase H and PNGase F Digestions

Immunoprecipitates were resuspended in 40 μl of a solution containing 100 mM sodium acetate, pH 5.5, 2% SDS, and 10% 2-mercaptoethanol. Samples were heated at 95°C for 3 min and the Pansorbin removed by centrifugation at 10,000 *g* for 3 min. 2 μl of endoglycosidase H (endo H) (1 U/ml; Miles Laboratories, Inc., Elkhart, IN) were added and the samples were incubated at 37°C for 14–16 h. To prepare the samples for electrophoresis, they were mixed with 2× gel sample buffer.

For peptide:*N*-glycosidase F (PNGase F) digestions, the solubilized [^{3}H]mannose-labeled proteins were chromatographed on Sephadex G-50 to remove residual free [^{3}H]mannose and the pooled void volume fractions were then digested for 16 h at 37°C with 1 mU of PNGase F in 0.4 ml as described (Roux et al., 1988). The digests were boiled and then rechromatographed on Sephadex G-50 to separate the released oligosaccharides from those still associated with the protein which remained in the void volume region (Freeze and Varki, 1986). The pooled oligosaccharides were desalted and used in the characterizations described below.

Analysis of Oligosaccharides

The released oligosaccharides were analyzed by quaternary ethyl amine (QAE)–Sephadex anion-exchange chromatography (Freeze et al., 1983) and by amine absorption HPLC using an Ax5 column eluted with a gradient of 35–65% water in acetonitrile (Freeze and Wolgast, 1986*b*). The procedures used for mild acid hydrolysis, alkaline phosphatase digestion, and solvolysis have been described previously (Freeze et al., 1983).

Membrane Association

HMW570 cells were pulse labeled with [^{35}S]methionine for 1 h. Cells were harvested by centrifugation and resuspended in water to 3 × 10^8 cells/ml, supplemented with M4 cells. The cells were then homogenized with a Dounce homogenizer and the postnuclear supernatants collected as above. The samples were split in two and to one was added 0.1% Triton X-100. The postnuclear supernatants were centrifuged at 100,000 *g* for 30 min in a Ti 50.3 rotor (Beckman Instruments, Inc., Fullerton, CA). The supernatants were removed from the membrane pellets, which were resuspended in water and centrifuged again. The membranes were dissolved in 0.5% Triton X-100 and prepared for immunoprecipitation.

Endocytosis Measurements

Logarithmically growing cells were collected by centrifugation (1,800 *g* for 3 min), and resuspended to a titer of 4 × 10^6 cells/ml in fresh TM medium containing 1 μCi/ml [^{3}H]dextran (sp act, 393 mCi/g). At various times, cells were centrifuged, washed three times with TM medium, resuspended in a small volume of 0.5% Triton X-100, and analyzed in a liquid scintillation counter. Percoll gradients were used to examine the properties of endosomal compartments. Cells were labeled with [^{3}H]dextran for 4 h as described above, collected by centrifugation, and subjected to Percoll gradient fractionation as previously described (Richardson et al., 1988). Samples were collected after centrifugation and analyzed for radioactivity, or assayed for α-mannosidase (lysosomal vesicle marker), α-glucosidase II (ER marker) (Borts and Dimond, 1981), and alkaline phosphatase activity (plasma membrane marker) (Parish and Pelli, 1974).

Results

Secretion of Lysosomal Enzymes from Wild-type and Mutant Cells

The mutant HMW570 is a member of a collection of over 70 mutants that are altered in secretion of lysosomal enzymes during growth and/or starvation conditions (Cardelli and Dimond, 1988; Dimond et al., 1983; Ebert, D. L., K. B. Jordan, R. L. Dimond, manuscript submitted for publication). As indicated in Fig. 1, a much greater percentage of the total

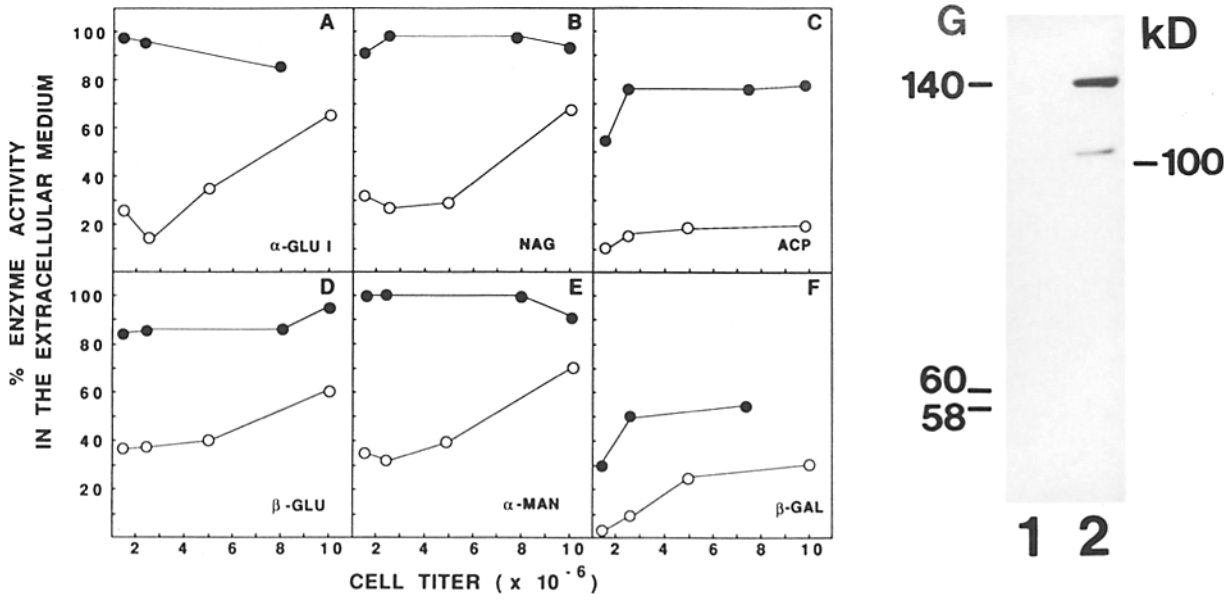

Figure 1. Secretion of lysosomal enzymes and steady-state radiolabeled forms of α-mannosidase and β-glucosidase. At the titers indicated in the figure, wild-type and mutant cells were collected by centrifugation and the following enzymes assayed in cell lysates and medium: α-glucosidase I (*A*), *N*-acetylglucosaminidase (*B*), acid phosphatase (*C*), β-glucosidase (*D*), α-mannosidase (*E*), and β-galactosidase 1 (*F*). (*G*) HMW570 cells were labeled with [^{35}S]methionine for three generations and α-mannosidase and β-glucosidase were immunoprecipitated using monoclonal antibodies added together. Lane *1* is the cellular sample and lane *2* is the secreted sample. The molecular masses in kilodaltons for wild-type α-mannosidase forms are indicated on the left, and for mature β-glucosidase forms on the right. (*A–F*) Wild-type (○) and mutant (●) strain.

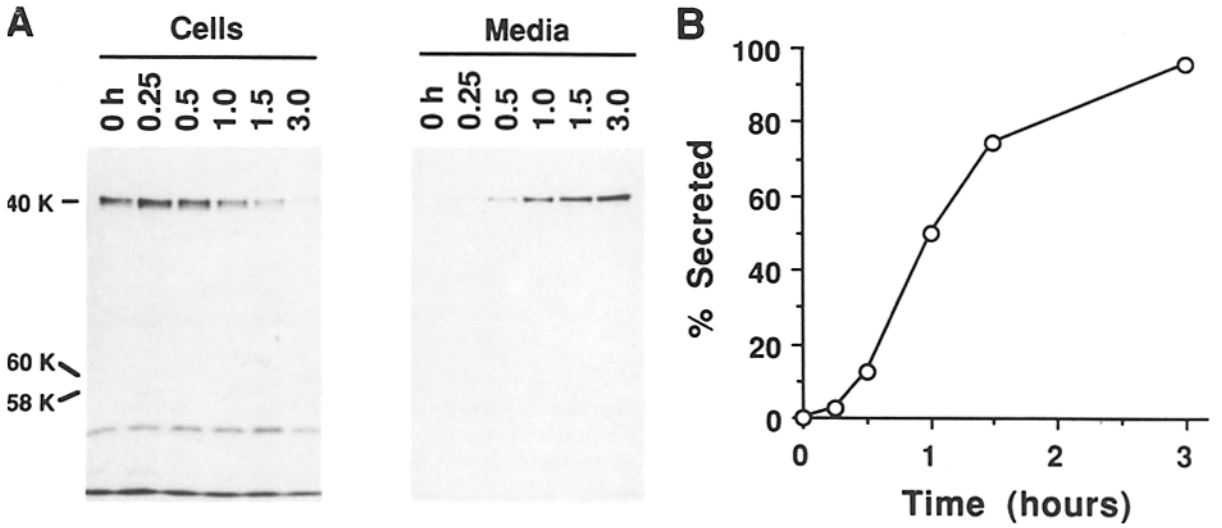

Figure 2. Processing and secretion of α-mannosidase in HMW570. Cells were pulse labeled for 30 min with [^{35}S]methionine and chased for the times indicated. (*A*) Radiolabeled α-mannosidase was immunoprecipitated and subjected to SDS-PAGE followed by fluorography. Cellular α-mannosidase is on the left (*Cells*), and secreted α-mannosidase is on the right (*Media*). (*B*) Percentage of labeled α-mannosidase found in the media at each time.

enzyme activity of six different acid hydrolases was found in the medium of HMW570 cultures at all cell densities compared to wild-type cells. For four of the enzymes examined (α-glucosidase I, *N*-acetylglucosaminidase, β-glucosidase, and α-mannosidase), the percent of total activity that was extracellular exceeded 80% compared to 30–40% in the wild-type strain Ax3. The observed increase in secretion of enzymes from wild-type cells normally occurs as cultures enter stationary phase at 10^7 cell/ml (Burns et al., 1981). In the next two sections we will demonstrate that the mutant oversecreted the lysosomal enzymes, α-mannosidase and β-glucosidase, in precursor form.

Biosynthesis of α-Mannosidase and β-Glucosidase in the Mutant Strain

In the mutant, HMW570, under steady-state radiolabeling conditions, only the 140-kD precursor form of α-mannosidase was present and nearly 100% of it was outside of the cell (Fig. 1 *G*, upper band). In contrast, under identical labeling conditions the wild-type precursor form makes up 10% of the extracellular α-mannosidase, but is in such small quantities inside the cell that it is undetectable (Mierendorf et al., 1985). The 60- and 58-kD mature forms are found in approximately equal amounts inside and outside cells in ex-

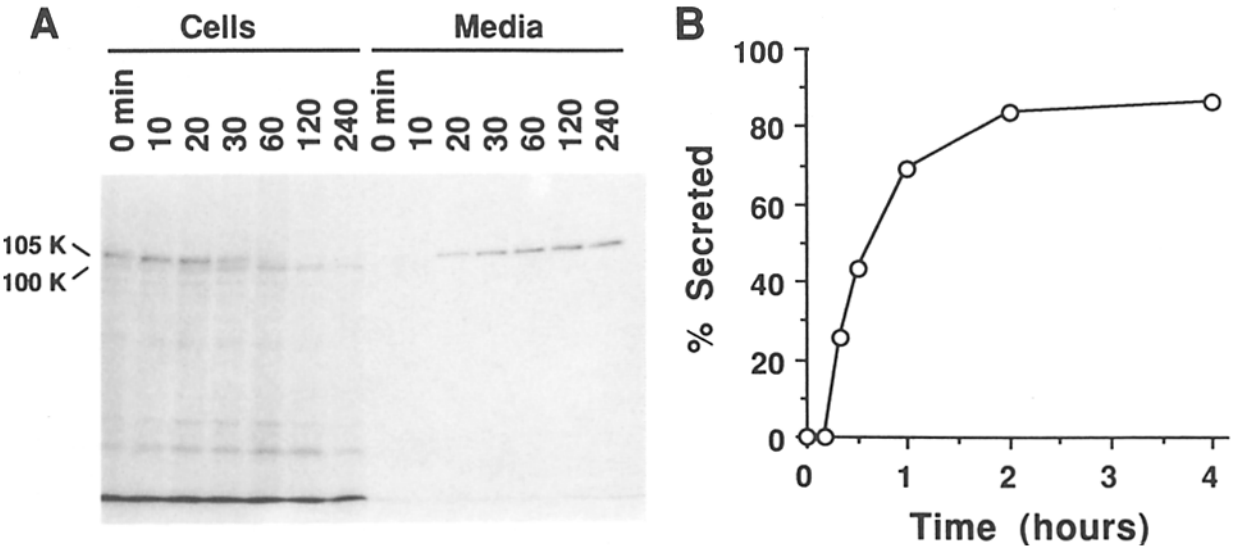

Figure 3. Processing and secretion of β-glucosidase in HMW570. Cells were pulse labeled for 15 min and chased for the times indicated. (*A*) β-Glucosidase was immunoprecipitated and subjected to SDS-PAGE and fluorography. Cellular β-glucosidase is shown on the left (*Cells*) and secreted β-glucosidase is on the right (*Media*). The molecular masses of β-glucosidase precursor (105 kD) and mature forms (100 kD) are indicated on the left. (*B*) Amount of labeled β-glucosidase precursor found in the media as a percentage of total labeled β-glucosidase.

ponentially growing cultures (Woychik et al., 1986). A pulse-chase experiment, in which mutant cells were pulse labeled with [^{35}S]methionine for 30 min and chased in the absence of label for various times, showed that the precursor was inefficiently processed to mature forms, and nearly all of the newly synthesized precursor was recovered outside of the cell (Fig. 2 *A*). Precursor began to be secreted from mutant cells 30 min into the chase period and secretion of mutant precursor did not plateau until 3 h into the chase (Fig. 2 *B*). The same experiment run in parallel with the wild-type strain showed that <5% of the α-mannosidase was secreted as precursor (data not shown) consistent with published observations (Mierendorf et al., 1985).

Steady-state labeling of HMW570 also revealed that, as for α-mannosidase, the major form of extracellular β-glucosidase observed was the 105-kD precursor (Fig. 1 *G*, lower band). Furthermore, in a pulse-chase experiment <20% of the precursor was converted to the 100-kD mature form (Fig. 3 *A*) and the remainder was secreted 30 min into the chase period (Fig. 3 *B*). The same experiment with Ax3 indicated that <1% of the β-glucosidase precursor is normally secreted (data not shown) consistent with the findings of Cardelli et al. (1986*a*).

Intracellular Movement of α-Mannosidase and β-Glucosidase in HMW570

HMW570 may oversecrete precursor polypeptides (along a constitutive pathway) because it is unable to correctly target these enzymes to lysosomes. Alternatively, the proteins could be directed to lysosomes but never processed to mature forms; moreover, secretion from this compartment might be unregulated in the mutant. To distinguish between these two possibilities, we used a pulse-chase protocol coupled with subcellular fractionation on discontinuous sucrose gradients to separate RER, the Golgi apparatus, and lysosomal vesicles of *D. discoideum* (Mierendorf et al., 1985; Cardelli et al., 1987). Using this technique, Mierendorf et al. (1985) showed that newly synthesized α-mannosidase precursor was transported from the RER to the Golgi complex with a half-time of 15–20 min, and 40 min into the chase period mature forms began to appear in the lysosomal fraction.

HMW570 cells were labeled for 20 min with [^{35}S]methionine and chased in fresh medium for 20 or 40 min. The cells were then broken in 0.25 M sucrose and organelles fractionated on a discontinuous sucrose gradient. As seen in Fig. 4 *A*, even after 40 min of chase, a time at which 25% of the

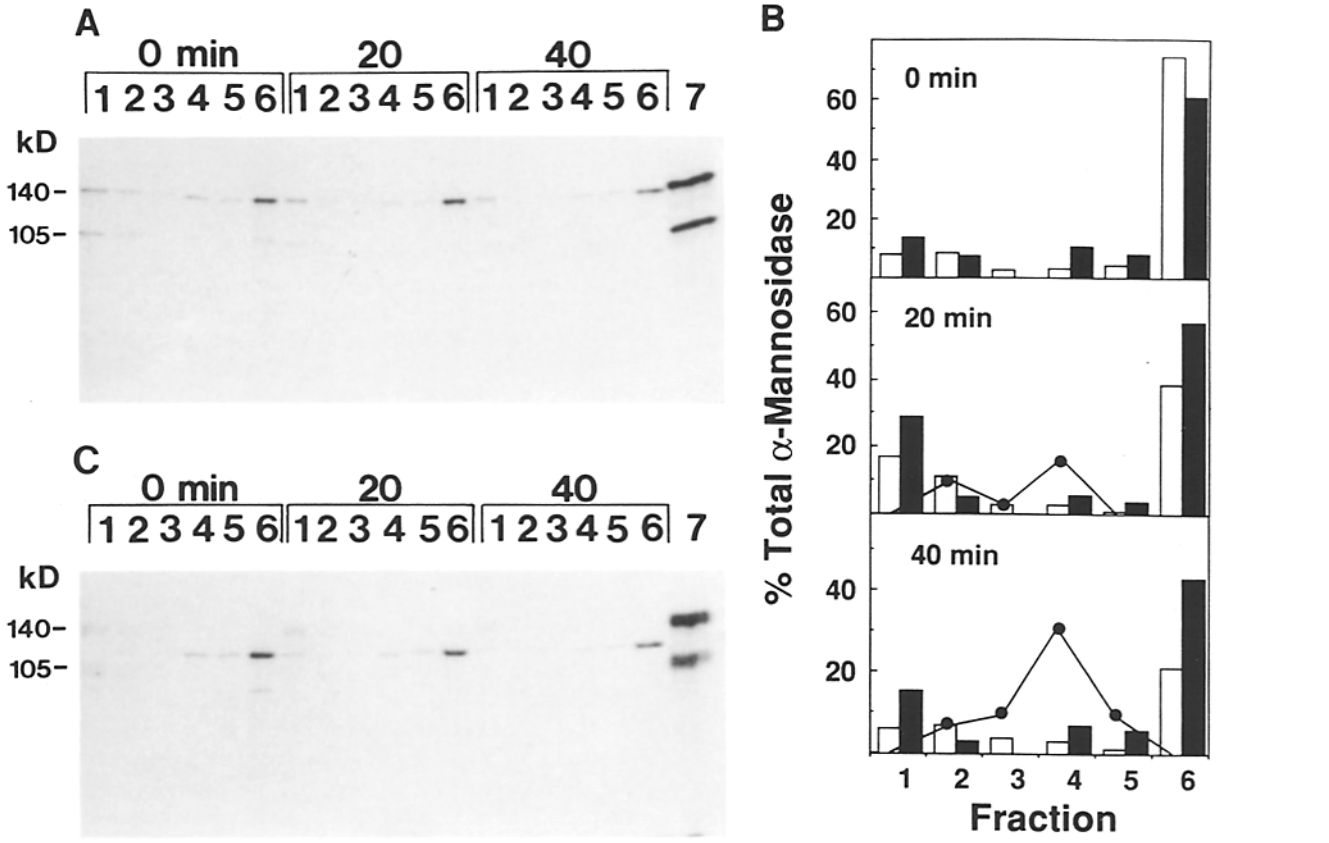

Figure 4. Intracellular transit of α-mannosidase and β-glucosidase in HMW570. Cells were pulse labeled for 20 min and chased for 0, 20, and 40 min. After labeling, cells were disrupted and postnuclear supernatants were fractionated on a discontinuous sucrose density gradient as described in Materials and Methods. Six fractions were collected starting from the top. α-Mannosidase and β-glucosidase were immunoprecipitated from each fraction and subjected to SDS-PAGE and fluorography. Lanes *1* and *2* correspond to the Golgi membranes (fractions *1* and *2*), lanes *3* and *4* correspond to the lysosomes (fractions *3* and *4*), lanes *5* and *6* correspond to the RER (fractions *5* and *6*), and lane *7* contains α-mannosidase and β-glucosidase polypeptides secreted after 40 min of chase. (*B*) The relative amounts of α-mannosidase found in each fraction. (*Solid bars*) HMW570 quantitated from *A*; (*open bars*) Ax3 precursor; (•) Ax3 mature forms. Ax3 cells were pulse labeled for 15 min. Samples identical to those shown in *A* were also treated with endo H as described in Materials and Methods before SDS-PAGE and fluorography (*C*). Molecular masses of Ax3 α-mannosidase (140 kD) and β-glucosidase (105 kD) precursors are indicated to the left of *A* and *C*.

mutant α-mannosidase precursor had been secreted (Fig. 2), and >50% of the wild-type α-mannosidase precursor would have been converted to the mature enzyme (Mierendorf et al., 1985; Fig. 4 *B*), no mature forms and only a small amount of the 140-kD mutant precursor was found in the lysosomal fraction (Fig. 4, *A* and *C*, lanes *3* and *4*). The precursor in these fractions was most likely contributed by low density RER membranes since the precursor had not acquired resistance to endo H (Fig. 4 *C*, 120-kD polypeptide observed in lanes *4–6*). The acquisition of resistance to endo H is a Golgi-associated event (Mierendorf et al., 1985), and both Golgi-localized and secreted mutant precursor were resistant to the action of endo H (Fig. 4 *C*, the 140-kD polypeptide observed in lanes *1*, *2*, and *7*). Chase times of up to 90 min have confirmed that no precursor was ever found in lysosomal vesicles (data not shown). In addition, no detectable β-glucosidase was targeted to lysosomes (Fig. 4, *A* and *C*, lower band).

An unexpected result from the fractionation experiments was that the rate of transit of α-mannosidase out of the RER was substantially slower in the mutant than in the wild-type cell. After a 20-min pulse, >70% of the precursor was still in the RER (Fig. 4 *B*, fractions *4–6*), and after a 20-min chase only an additional 10% had moved to the Golgi apparatus. After a 40-min chase, considering ~25% of the precursor had been secreted (see Fig. 2 *B*), >50% of the radiolabeled precursor still remained in the RER. In contrast, in wild-type cells by 40 min of chase ~20% of the α-mannosidase precursor remains in the ER (Fig. 4 *B*; Cardelli et al., 1986*a*). The fact that relatively little precursor ever accumulated in the Golgi apparatus, indicated that transit of α-mannosidase out of the Golgi system to the cell surface was not delayed in the mutant. In summary, the delayed secretion of the α-mannosidase precursor from HMW570 (Fig. 2) can be accounted for in part by the retention of this protein in the ER.

To substantiate the fractionation results, a pulse-chase experiment was performed, and immunoprecipitated α-mannosidase was treated with endo H. In Ax3, by 20 min of chase (after a 15-min pulse), 65% of the α-mannosidase precursor has become endo H resistant (Cardelli et al., 1986*a*). In contrast, at this time only 10% of the mutant cellular precursor was endo H resistant (data not shown; see also Fig. 4 *C*). This confirms that transit from the site of synthesis (RER) to the site of acquisition of endo H resistance (the Golgi apparatus) was much slower in the mutant than in the wild-type precursor.

Mutant Lysosomal Enzyme Precursors Are Membrane Associated

We have previously demonstrated that the α-mannosidase and β-glucosidase precursors are membrane bound while mature forms are soluble (Cardelli et al., 1986*a*; Mierendorf et al., 1985). Fig. 5 indicates that 95% of pulse-labeled α-mannosidase and β-glucosidase precursors from HMW570 were still associated with membranes after breakage of cells in water; in contrast, 70% of the mature forms in Ax3 were soluble (Fig. 5, lanes *1*, *2*, *5*, and *6*). Furthermore, disruption of membranes by the detergent Triton X-100 before centrifugation resulted in recovery of the precursors in the supernatant fraction (Fig. 5, lanes *3*, *4*, *7*, and *8*). This suggests that the precursors were pelleted by centrifugation due to interaction with the membranes, and not because of aggregation.

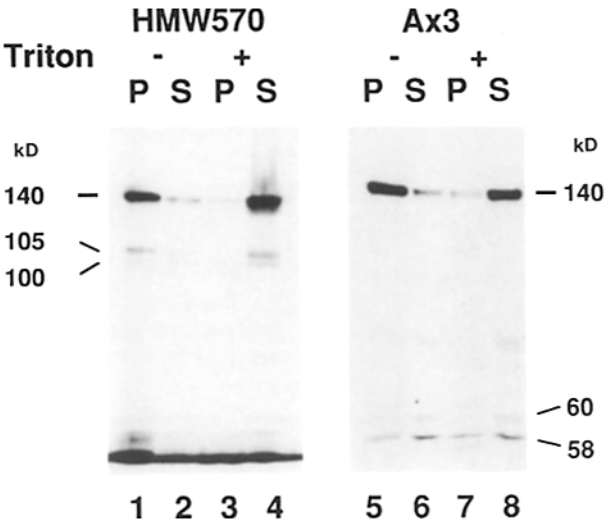

Figure 5. α-Mannosidase and β-glucosidase precursors are membrane associated in HMW570. Cells were labeled for 1 h and broken in water with a Dounce homogenizer. After a 30-min incubation on ice, half of the samples were adjusted to 0.5% Triton X-100 (lanes *3*, *4*, *7*, *8*). Membrane-bound proteins (lanes *1* and *5*) and aggregated proteins (lanes *3* and *7*) were separated from soluble proteins (lanes *2*, *4*, *6*, and *8*) by high speed centrifugation. α-Mannosidase and β-glucosidase were immunoprecipitated from the pellet (*P*) and supernatant (*S*) fractions of HMW570 (left panel) and α-mannosidase alone was precipitated from Ax3 (right panel). Molecular masses of α-mannosidase precursor (140 kD) and mature (60 and 58 kD) forms, and β-glucosidase precursor (105 kD) and mature (100 kD) forms are indicated.

Release of N-linked Oligosaccharide Chains from α-Mannosidase Polypeptides

[^{3}H]Mannose-labeled α-mannosidase from Ax3 and HMW-570 were immunoprecipitated and analyzed by SDS-PAGE (Fig. 6). The [^{3}H]mannose-labeled, 20-kD glycopeptide (Fig. 6, lane *4*) is derived from cleavage of the 80-kD α-mannosidase intermediate which generates the 60-kD mature form (results not shown).

The immunoprecipitates were solubilized in SDS, and isolated from the void volume region of a Sephadex G-50 column. They were then digested with protease-free PNGase F to remove N-linked oligosaccharides and rechromatographed on Sephadex G-50 to separate the released chains from those still bound to the protein (Fig. 7). The oligosaccharides from the precursor of HMW570 were totally sensitive to digestion under these conditions (Fig. 7 *A*), while those from the precursor form of wild-type (Fig. 7 *B*) and the mature forms of both strains (Fig. 7, *C* and *D*) were partially resistant. Redigestion of the pooled, unreleased material from the wild-type mature forms released an additional 10% of the label (Fig. 7 *E*). However, when mature α-mannosidase from the wild-type was first digested using a 20-fold greater concentration of PNGase F, ~85% of the total ^{3}H was released (Fig. 7 *F*). These results suggest that the oligosaccharides on

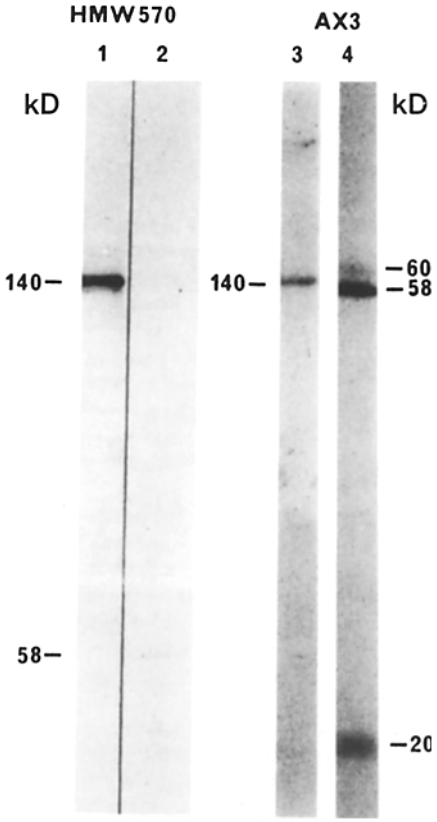

Figure 6. SDS-PAGE of [^{3}H]mannose-labeled α-mannosidase from wild-type and HMW570 cells. Wild-type and HMW570 cells were labeled with [^{3}H]mannose and the mature and precursor forms of secreted mannosidase were isolated by sequential immunoprecipitation with antibody 6H10 (mature form specific) or antibody 2H9 (mature and precursor forms) as described in Materials and Methods and analyzed on a 7.5% polyacrylamide gel. Strain HMW570, antibody 2H9 (lane *1*, 6,000 dpm) and antibody 6H10 (lane *2*, 2200 dpm) precipitations (40-d exposure). Wild-type strain, antibody 2H9 (lane *3*, 4,000 dpm, 30-d exposure) and 6H10 (lane *4*, 12,000 dpm, 9-d exposure) precipitations. Molecular masses in kilodaltons are shown.

the precursor are normally modified to a PNGase F–resistant form and that the precursor of HMW570 was not similarly modified.

Characterization of Neutral and Anionic N-Linked Oligosaccharides on Mutant and Wild-type α-Mannosidase Polypeptides

Dictyostelium lysosomal enzymes contain neutral and anionic N-linked oligosaccharides due to the presence of multiple residues of Man-P-OCH_3 and mannose-6-sulfate (Freeze, 1986). Fractionation of the oligosaccharides by QAE–Sephadex chromatography (Fig. 8) shows the patterns from both wild-type and HMW570 precursor forms are nearly the same as those from the mature form of the wild-type enzyme. This result suggests that the releasable oligosaccharides were similarly modified in all three species.

To determine the relative contribution of each of the anionic groups, the sulfate was cleaved by solvolysis in DMSO and the products again analyzed on QAE-Sephadex. The residual charge is now due only to Man-P-OCH_3. The results shown in Fig. 8, *D–F*, indicate that the proportion of oligosaccharides modified by the addition of one, two, or three phosphodiesters or only sulfate esters are nearly the same. Similar analysis of pools of various charged fractions shown in Fig. 8, *A–C*, revealed that the precursors from both strains are also similar to the mature form of the wild-type (data not shown). Furthermore, alkaline hydrolysis showed that essentially all of the mannose-6-phosphate was found as an acid-resistant phosphodiester (Man-P-OCH_3) in all three species. Finally, the size of the neutral oligosaccharides found in the precursor form of HMW570 were the same as those from the mature forms of the wild-type cells.

These results suggest that all of the known N-linked oligosaccharide modifications occur on the precursor form and that, except for the partial PNGase resistance of HMW570 precursor, the sugar modifications are normal in the precursor forms of the wild-type and mutant strains.

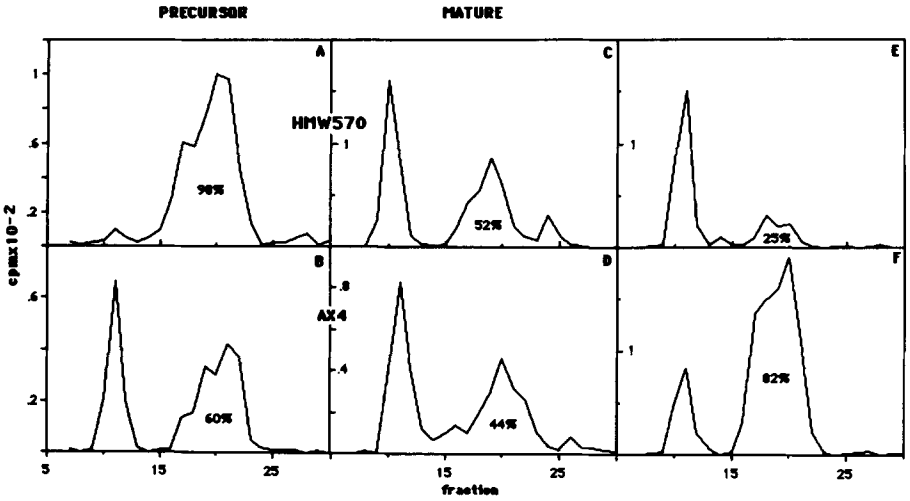

Figure 7. Sephadex G-50 analysis of [^{3}H]mannose-labeled α-mannosidase after PNGase F digestion. Immunoprecipitated precursor and mature forms of α-mannosidase from wild-type and HMW570 cells were digested with PNGase F (7 mU/ml) as described in Materials and Methods (*A–E*). The digests were then run on Sephadex G-50 in 0.2% SDS and 10 mM Tris-HCl, pH 7.2, and aliquots were counted. (*A*) Precursor form of HMW570; (*B*) precursor form of wild-type α-mannosidase; (*C*) mature form of HMW570; (*D*) mature form of wild-type α-mannosidase; (*E*) redigestion of the unreleased material (void volume region) from *D*; (*F*) first digestion of mature form of wild-type using PNGase F at 156 mU/ml. Void volume occurs at fractions 10–12 and mannose elutes at fractions 27–29.

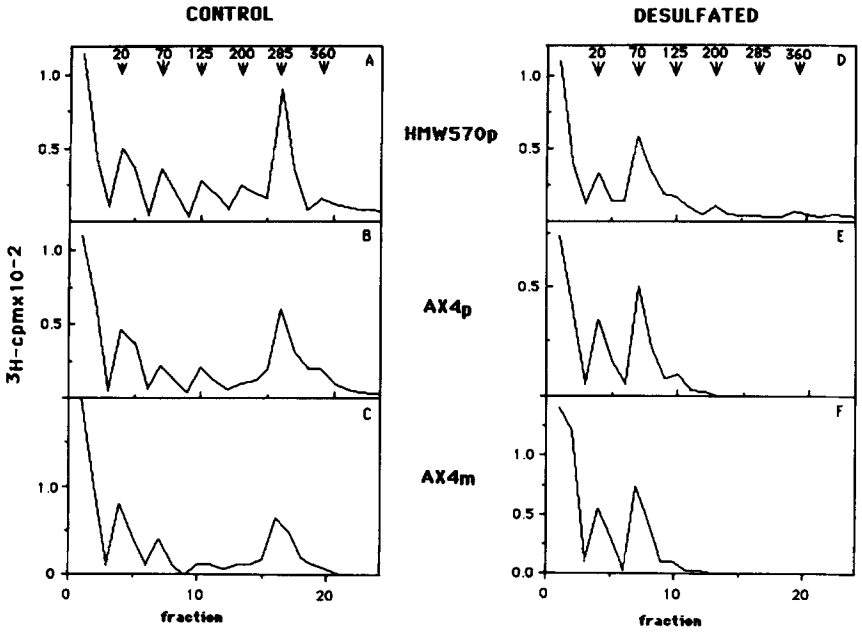

Figure 8. QAE-Sephadex analysis of PNGase F released N-linked oligosaccharides. The released oligosaccharides were pooled, desalted on Sephadex G-25, and analyzed on 2-cm QAE-Sephadex columns using 3 × 1.5-ml washes of 0, 20, 70, 125, 200, 285, and 400 mM NaCl in 2 mM Tris base. These concentrations elute molecules with no, one, two, three, four, five, and six negative charges, respectively. *A*, *B*, and *C* are HMW570 precursor, wild-type precursor, and wild-type mature, respectively. *D*, *E*, and *F* are identical samples analyzed after solvolysis to remove sulfate esters.

HMW570 Is Defective in Endocytosis

Previous genetic and biochemical studies have indicated that the intracellular lysosomal enzyme transport pathway may merge with the endocytic pathway (von Figura and Hasilik, 1986; Roff et al., 1986; Robbins et al., 1984; Diment et al., 1988; Griffiths et al., 1988; Brown et al., 1986; Banta et al., 1988). To begin to analyze the endocytic pathway in *Dictyostelium*, we pulse labeled growing cells for 15 min with the fluid-phase marker, [^{3}H]dextran, followed by a chase period in fresh medium in the absence of radiolabeled dextran. Cells were disrupted by homogenization and organelles were separated by centrifugation on self-forming Percoll gradients. As indicated in Fig. 9 *A*, after the pulse period, >65% of the fluid-phase marker was contained in light vesicles with a density (p = 1.04 g/ml) similar to the Golgi complex, ER, and plasma membrane (Fig. 9 B). Based on results of previous studies, we conclude that these low density vesicles are endosomes (Merion and Sly, 1983). Vesicles containing the fluid-phase marker increased in density very rapidly ($t_{1/2}$ = 5 min) during the chase period consistent with the maturation of light endosomes to dense secondary lysosomes (see Fig. 9 *B*). Thus, internalized fluid-phase markers in *Dictyostelium* apparently follow intracellular endocytic pathways analogous to those described for mammalian cells.

Fig. 10 *A* indicates the rate of accumulation of [^{3}H]dextran in wild-type and HMW570 cells. The fluid-phase marker accumulated linearly for 2 h in wild-type cells. This was followed by a steady decrease in the rate of uptake between 2 and 8 h, and by 8 h no further accumulation was observed suggesting that influx and efflux were equal. This same general accumulation pattern was observed for the mutant HMW570. However, the rate of accumulation at all times was approximately one-half that of wild-type cells, and the mutant accumulated only one-half the amount of [^{3}H]dextran.

Fig. 10 *B* indicates the rate of release of [^{3}H]dextran from wild-type and mutant cells after a labeling period of 4 h. Both wild-type and mutant cells secreted the fluid-phase marker very rapidly ($t_{1/2}$ of 75 and 55 min, respectively) and to the same extent; by 6 h, only 20-30% of the dextran remained associated with cells. This result indicates that the decreased accumulation of labeled dextran in HMW570 is not due to major changes in membrane flow.

Fig. 11, *A* and *B*, indicates the distribution of the fluid-phase marker on Percoll gradients after centrifugation of wild-type and mutant cell extracts prepared after a labeling period of 4 h. [^{3}H]Dextran distributed as three peaks after fractionation of organelles from wild-type cells (Fig. 11 *A*). The top peak represented label released from ruptured vesicles, the low density peak (p = 1.04 g/ml) most likely corresponded to endosomes (see Fig. 9), while the bottom peak (p = 1.09 g/ml) coincided in position with the peak of the lysosomal marker enzyme, acid phosphatase (Fig. 11 *C*). A distinctly different profile of labeled dextran was observed after fractionation of organelles from HMW570 cells. Fig. 11 *B* indicates that between 30 and 35% of the total intracellular dextran resided in a compartment similar in density to endosomes, while <7% accumulated in dense vesicles (fractions *1–5*). Fig. 11, *C* and *D*, represents the distribution of the lysosomal marker enzyme, acid phosphatase, on Percoll gradients after fractionation of cell extracts prepared from wild-type (Fig. 11 *C*) and mutant (Fig. 11 *D*) cells. In wild-type cells an equal percentage (35%) of the total acid phosphatase activity was distributed in both light organelles (p = 1.04 g/ml) and dense organelles (most likely secondary lysosomes). In contrast, only 20% of the total residual acid phosphatase activity remaining in HMW570 cells was in dense lysosomes while 53% of the enzyme activity was in vesicles corresponding in density to endosomes. Thus, the mutant is altered not only in the extent and the kinetics of accumulation of a fluid-phase marker, but also appears inefficient in the generation of dense secondary lysosomes.

Discussion

Two forms of lysosomal α-mannosidase and β-glucosidase are secreted from *D. discoideum* (Mierendorf et al., 1983; Pannell et al., 1982). Newly synthesized precursors are

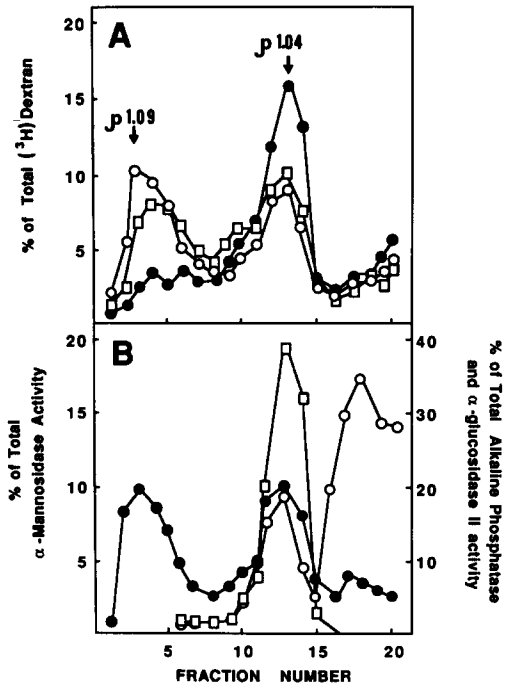

Figure 9. Uptake and intracellular transport of the fluid-phase marker, [^{3}H]dextran, in wild-type cells. Growing cells were pulse labeled with [^{3}H]dextran for 20 min (●), washed three times, and resuspended in fresh growth medium without dextran to initiate the chase period. Cells were harvested at 10 (□) and 30 min (○) into the chase period, broken by homogenization, and postnuclear supernatants fractionated on Percoll gradients as described in Materials and Methods. *A* indicates the distribution of [^{3}H]dextran, while *B* indicates the distribution of the enzymes α-mannosidase (●, lysosomal marker), α-glucosidase I (□, ER/Golgi marker), and alkaline phosphatase (○, plasma membrane marker).

secreted constitutively, while mature forms in lysosomes begin to be secreted after a 2–3-h lag (Mierendorf et al., 1985; Wood et al., 1983; Cardelli et al., 1986*a*). Secretion of mature forms of the enzymes occurs at a low rate during growth, and is significantly stimulated after starvation of cells (Dimond et al., 1981). The mutant strain described in this report, HMW570, secretes nearly 90% of its α-mannosidase and β-glucosidase activities. This lysosomal enzyme oversecretion could be the result of (*a*) misregulation of the lysosomal secretory system during growth, or (*b*) misdirection of a greater percentage of the lysosomal enzyme precursor down the constitutive secretory pathway. The first possibility predicts that secreted enzyme would be coming from the lysosomal vesicles and would, therefore, be of the mature form. This was not the case since the oversecretion in both strains can be accounted for by increased precursor secretion. Furthermore, subcellular fractionation studies indicated that the precursor polypeptides never passed through the lysosomes.

Precursor missorting and oversecretion could be the result of any of a number of factors. Yeast cells, which have been genetically engineered to overproduce vacuolar carboxypeptidase Y, secrete >50% of the enzyme in the form of the precursor, perhaps because a targeting receptor may be saturated (Stevens et al., 1986). Human fibroblasts from patients with either I-cell disease or pseudo-Hurler polydystrophy are missing the mannose-6-phosphate targeting signal (Reitman et al., 1981) and, consequently, secrete precursor forms of lysosomal enzymes (Hasilik and Neufeld, 1980*b*; Mellman et al., 1986). Mutant Chinese hamster ovary cells containing defective mannose-6-phosphate receptors also secrete lysosomal enzyme precursors (Robbins and Myerowitz, 1981) as do human fibroblasts incubated in the presence of either ammonia or chloroquine (Hasilik and Neufeld, 1980*b*), which raises the intralysosomal pH and effectively blocks the recycling of receptors (Gonzalez-Noriega et al., 1980). Finally, mammalian cell mutants have been described that are defective both in endocytosis and sorting of lysosomal enzymes (Roff et al., 1986; Robbins, 1988).

In contrast to the genetically engineered yeast cells, HMW570 did not overproduce either α-mannosidase or β-glucosidase. In fact, during growth HMW570 only has, on

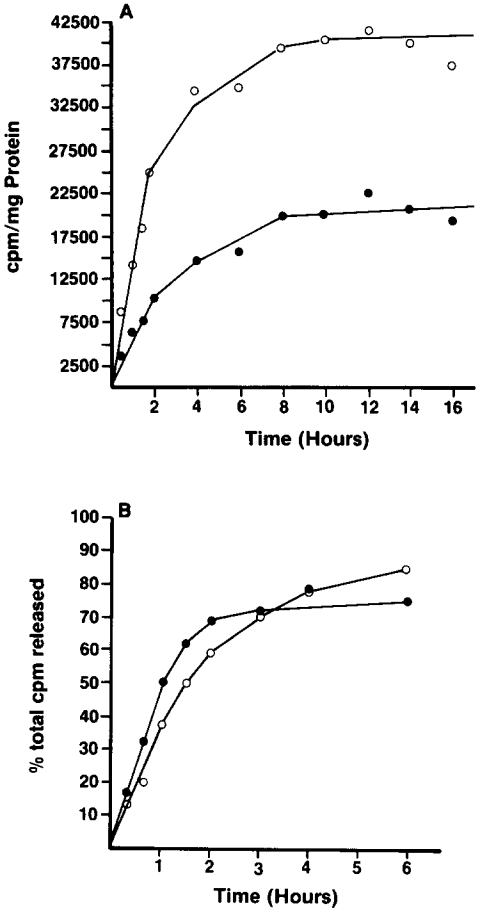

Figure 10. Rate of endocytosis and exocytosis of [^{3}H]dextran in wild-type and mutant cells. Exponentially growing wild-type (○) and HMW570 (●) cells were recovered by centrifugation and resuspended at 4 × 10^6 cells/ml in fresh growth medium containing [^{3}H]dextran at 1 μCi/ml. At the times indicated in the figure (*A*), cells were centrifuged, washed, solubilized in Triton X-100, and cell-associated radioactivity determined using a liquid scintillation counter. To determine exocytosis rates, cells were labeled with dextran for 4 h, washed, and resuspended in fresh growth medium. At the indicated times (*B*), cells were pelleted by centrifugation and the dextran that was released into the medium determined using a liquid scintillation counter.

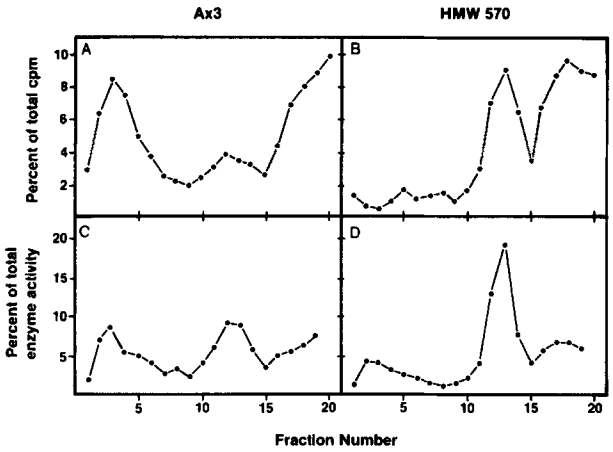

Figure 11. Intracellular distribution of [^{3}H]dextran and acid phosphatase in HMW570 and wild-type cells. Wild-type (*A* and *C*) and mutant (*B* and *D*) cells were labeled with [^{3}H]dextran for 4 h, and intracellular organelles fractionated on Percoll gradients as described in Materials and Methods. Fractions were analyzed for radioactivity (*A* and *B*) and acid phosphatase activity (*C* and *D*).

the average, 14.0 ± 1.0 U total of α-mannosidase activity both inside and outside of the cell per 10^7 cells, and 13.3 ± 1.3 U of β-glucosidase. In contrast, Ax3 has 47.6 ± 3.7 U of α-mannosidase and 13.5 ± 1.4 U of β-glucosidase. It is still possible that the mutant cells overproduce the enzyme but that most of it is enzymatically inactive. However, measurements of the relative synthetic rates for both enzymes indicates no difference between wild-type and mutant cells (results not shown). Thus, missorting is most likely not due to overproduction of precursor polypeptides and saturation of sorting receptors as proposed for yeast.

The N-linked oligosaccharides of *Dictyostelium* wild-type lysosomal enzymes are normally modified by minimal removal of mannose residues and the addition of *N*-acetylglucosamine, sulfate esters, and phosphodiesters (Freeze, 1986). The functional significance of the modifications is not known. We, therefore, compared the N-linked oligosaccharides released from α-mannosidase precursor and mature forms isolated from mutant and wild-type cells. Unlike I-cell lysosomal enzymes, all four forms showed remarkable similarity to each other in terms of the size, charge distribution, amount of sulfation and phosphorylation, and state of esterification of the phosphate esters. All of the oligosaccharide modifications probably occur on the precursor before cleavage to the mature form. It is unlikely, therefore, that the failure to correctly target the lysosomal enzymes in HMW570 is due to the lack of any of the known sugar modifications.

About one-half of the oligosaccharides in the wild-type mature and precursor forms become resistant to facile digestion with PNGase F, in contrast to HMW570 where all the oligosaccharides are released from the precursor. This may be due to an unknown modification of the oligosaccharide or the peptide, but it is more likely to be an oligosaccharide modification for two reasons. First, the protein is totally denatured in SDS before digestion; and secondly, PNGase F digestion under the facile conditions is sufficient to cleave the great majority of N-linked oligosaccharides from several mammalian cell lines (Freeze and Varki, 1986). It remains possible that the modification is important in the sorting of lysosomal enzymes although secreted precursor from wild-type cells still contains it. Alternatively, the precursor in HMW570 is missorted before passing through the compartment where the modification occurs.

No mannose 6-phosphate targeting receptors have yet been found in *D. discoideum*, even though lysosomal enzymes from this organism bind with high affinity to mammalian receptors. However, α-mannosidase and β-glucosidase precursors are membrane associated, and become soluble after proteolytic cleavage and arrival in the lysosomes (Mierendorf et al., 1985; Cardelli et al., 1986*a*). We have previously speculated that this membrane association may be important in lysosomal enzyme targeting. However, α-mannosidase and β-glucosidase precursors in the mutant was membrane associated, indicating that membrane association alone is not sufficient for targeting. We have not yet defined the physical nature of this interaction which could conceivably involve specific receptors. Therefore, it is possible that the physical association between precursors and membranes in the mutant differs from wild-type cells in a way which may profoundly influence proper sorting.

Our most intriguing finding was that the lysosomal enzyme sorting mutant HMW570 was defective in endocytosis. Both the rate and extent of accumulation of a fluid-phase marker was reduced 50% in the mutant. Furthermore, a much smaller percentage of the fluid-phase marker resided in dense vesicles (most likely lysosomes) compared to wild-type cells after a long pulse labeling. Finally, the residual acid phosphatase activity remaining in HMW570 was predominately in vesicles with the density of endosomes or Golgi membranes; very little activity was detected in secondary lysosomes.

HMW570 is phenotypically similar to many of the endocytic mutants previously described by others (for review see Robbins, 1988). Many of these cell lines also missort and oversecrete lysosomal enzymes perhaps due to the inability to acidify endosomes. As a result of this increase in pH, ligands may not be released from receptors leading to a depletion of unoccupied receptors in the Golgi complex, and the missorting of newly synthesized lysosomal enzyme precursors. In contrast, defects in acidification most likely do not account for the phenotypic properties of HMW570 for the following reasons. First, raising the lysosomal/endosomal pH to 6.4 using the weak base ammonium chloride did not result in a significant increase in the secretion of newly synthesized lysosomal enzyme precursors from wild-type cells (Cardelli et al., 1989). Second, direct measurements of intravesicular pH in HMW570 using the spectrofluorometric method of Ohkuma and Poole (1978) revealed that mutant and wild-type cells both maintained a lysosomal/endosomal pH of 5.4 (results not shown). Finally, in vitro acidification assays using FITC–dextran-loaded endosomes and lysosomes from the mutant and wild-type strains revealed no difference in the rate of ATP-dependent quenching of fluorescence.

Taken together, our results suggest the targeting block is in a portion of the lysosomal pathway that is shared with the endocytic pathway, but is independent of the acidification of lysosomal and endosomal vesicles. We believe, therefore, that the mutation in HMW570 is preventing the formation of secondary lysosomal vesicles. This is supported by the absence of residual acid phosphatase activity, as well as a marker for fluid-phase endocytosis in a region of Percoll den-

sity gradients where lysosomal enzymes would normally reside. Phenotypically, HMW570 resembles the yeast endocytosis mutant *end* 1 described by Chvatchko et al. (1986). This mutant, which is a member (*vpt* 11) of a large class of yeast vacuole protein localization mutants described by the Stevens and Emrs laboratories (Banta et al., 1988; Rothman and Stevens, 1986), is also defective in the formation of the vacuole.

We can only speculate at the present time as to how an apparent block in the endosomal pathway could lead to lysosomal enzyme missorting. After leaving the Golgi complex, lysosomal enzyme precursors most likely move to endosomal compartments where the first proteolytic cleavages occur, an event that may be important in the sorting of enzymes in *Dictyostelium* (Richardson et al., 1988). After cleavage, the intermediate forms may be collected and packaged into vesicles that bud from endosomal compartments and mature into dense secondary lysosomes where completion of proteolytic processing occurs (Cardelli et al., 1989). HMW570 may be defective in the proteolysis step, an event that has been shown by others (Opresko and Karpf, 1987) to be important in the maturation and fusion of endosomes with yolk platelets (lysosomal-like vesicles in *Xenopus* oocytes). Thus, uncleaved precursors may reside in endosomes longer than normal, resulting in missorting due to incorporation of these polypeptides into vesicles which recycle from endosomes to the cell surface. Alternatively, the mutation in HMW570 may inactivate a gene product involved in both the targeting of precursors to endosomes and the maturation of endosomes to dense lysosomes. Currently our studies are aimed at determining the role the endocytic pathway plays in the sorting and proteolysis of lysosomal enzymes.

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